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Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors

(nodules/nodule-specific genes/development)

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ABSTRACT **_Rhizobium nod_ genes are essential for root hair deformation and cortical cell division, early stages in the development of nitrogen-fixing root nodules. Nod⁻ mutants are unable to initiate nodules on legume roots. We observed that _N_-(1-naphthyl)phthalamic acid and 2,3,5-triiodobenzoic acid, compounds known to function as auxin transport inhibitors, induced nodule-like structures on alfalfa roots. The nodule-like structures (pseudonodules) were white, devoid of bacteria, and resembled nodules elicited by _Rhizobium meliloti_ exopolysaccharide (_exo_) mutants at both the histological and molecular level. Two nodulin genes, ENOD2 and Nms-30, were expressed. RNA isolated from the nodule-like structures hybridized to pGmENOD2, a soybean early nodulin cDNA clone. RNA isolated from roots did not hybridize. We determined by _in vitro_ translations of total RNA that the alfalfa nodulin transcript Nms-30 was also expressed in the nodule-like structures. The late expressed nodulin genes, such as the leghemoglobin genes, were not transcribed. Because _N_-(1-naphthyl)-phthalamic acid and 2,3,5-triiodobenzoic acid induce the development of nodules on alfalfa roots, we suggest that the auxin transport inhibitors mimic the activity of compound(s) made upon the induction of the _Rhizobium nod_ genes.**

The nodulation (*nod*) genes of *Rhizobium* play an essential role in the induction of nodules on the roots of leguminous plants. The importance of the *nod* genes has been demonstrated in at least two ways. First, it has been shown that *Rhizobium* that have mutated common *nod* genes, *nodABC*, lose the ability to curl root hairs (1) as well as to initiate cellular divisions within the root cortex. Cortical cell divisions mark the beginning of root nodule formation (2). Second, by transferring the *nod* region of *Rhizobium meliloti* or *Rhizobium leguminosarum* to *Agrobacterium tumefaciens*, *Agrobacterium* transconjugants acquire the ability to form nodules on alfalfa and *Vicia*, respectively (3, 4).

Although the *nod* genes are required for the earliest stages of nodule development, it is not known how the *nod* gene products induce root hair curling and cortical cell divisions. The gene products of *nodA* and *nodC* have been immunolocalized in the rhizobial cytosol and outer membrane, respectively (5, 6). John *et al.* (7) have proposed that the *nodC* gene product aids in the transfer of growth factors from *Rhizobium* to the plant. Studies of *nod* gene regulation (cf. publications in ref. 8) have indicated that flavones—e.g., luteolin, apigenin, and 7,4′-dihydroxyflavone—are essential for the expression of *nod* genes in *Rhizobium* (9–11). However, the function(s) of the *nod* gene products has yet to be determined.

Plant hormones have been suggested as playing a critical role in nodule development since the first report of auxin involvement in pea root nodulation by Thimann (12). *Rhizobium* produces auxins, cytokinins, and gibberellin-like substances (cf. reviews in refs. 13 and 14), but none of the common *nod* genes has been shown to be involved in plant hormone synthesis. Libbenga *et al.* (15) studied the initiation of cortical cell divisions in legume root tissues in response to auxins and cytokinins in an effort to relate hormone levels to nodule formation. Bauer *et al.* (16) demonstrated that the cytokinin benzyladenine, as well as unknown diffusible substances produced by homologous but not heterologous rhizobia, induced cortical cell divisions in a number of legumes. Despite these efforts, a precise role for the plant hormones, whether endogenous or introduced by the bacterial symbionts, in nodule initiation and development remains undefined.

There are reports on the formation of nodule-like structures or pseudonodules on roots by treatment with the cytokinins (17, 18). However, the internal structure of these cytokinin-induced pseudonodules differed from that of *Rhizobium*-induced nodules. Substituted benzoic acids, chemicals that presumably modify auxin levels in the plant (19, 20), also induce nodule-like structures on legume roots (refs. 21 and 22; J.G.T., unpublished results).

We found that the histology of the nodule-like structures induced by the auxin transport inhibitors *N*-(1-naphthyl)-phthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) closely resembled that of *Rhizobium*-induced legume nodules. We studied these nodule-like outgrowths to determine their relation to root nodules initiated by *Rhizobium*. In particular, we found that the pseudonodules were very similar to nodules induced by exopolysaccharide (*exo*) mutants of *R. meliloti* (23). *R. meliloti exo* mutants, although unable to penetrate alfalfa root tissue, are capable of inducing cortical cell divisions and nodule development. Apparently, the *Rhizobium* mutants produce a signal, which is expressed within the root cortex, at a distance removed from the source of the signal.

While more than 20 nodulins have been identified in wild-type-induced alfalfa nodules, only two nodulin genes have been shown to be expressed in nodules induced by *exo* mutant *R. meliloti*. They are the Nms-30 gene, identified by *in vitro* translation of nodule RNA, and a gene in alfalfa comparable to the soybean early nodulin gene ENOD2. Transcripts for other nodulin genes, such as the Lb genes, are not present in the *exo* mutant-induced nodules (24–26).

We used cloned early nodulin DNAs as probes to investigate gene expression of the nodule-like structures induced by NPA and TIBA. Our goal was to determine whether the pseudonodules elicited by the auxin transport inhibitors

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Abbreviations: NPA, *N*-(1-naphthyl)phthalamic acid; TIBA, 2,3,5-triiodobenzoic acid.

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resembled *Rhizobium*-induced nodules at the molecular level.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Seeds of *Medicago sativa* L. cv. Iroquois (alfalfa) were sterilized as described (27) and sown in autoclaved plastic pans containing sterilized perlite covered with 2 cm of sterile vermiculite. The pans were watered with 1.5 liters of Jensen's medium (28) without nitrogen at the time of sowing. The seedlings were grown under conditions of 16 hr, 21°C day/8 hr, 19°C night. Two-week-old seedlings were watered with an additional 1.5 liters of Jensen's medium containing dilutions of the various benzoic and phthalamic acid derivatives with 5–40 mM nitrate or without nitrate. Distilled water was used for watering the seedlings between and after treatments.

Additional plants were grown in test tubes as described by Meade *et al.* (27). Benzoic acids were applied as aqueous solutions to the seedling roots 2 weeks after planting.

Alfalfa seedlings were inoculated with wild-type or *exo* mutant *R. meliloti* as described (25).

Benzoic and Phthalamic Acid Derivatives. TIBA (Sigma) was dissolved in ethanol and diluted to 5×10^{-5} M in Jensen's medium containing nitrate. A 10^{-2} M stock solution of NPA [2-(1-naphthalenylaminocarbonyl)benzoic acid] (U.S. Rubber) was prepared in an alkaline aqueous solution and diluted to 10^{-5} M before application to plants.

RNA Isolation. Roots and their nodule-like structures were harvested separately 3–4 weeks after treatment, frozen immediately in liquid nitrogen, and stored at −70°C. RNA was isolated separately from the frozen roots and nodule-like structures as described (29).

***In Vitro* Translations and RNA Transfer Blots.** Total RNA was translated in a rabbit reticulocyte system (29) or prepared for RNA transfer blots as described by Maniatis *et al.* (30). The RNA for transfer blots was transferred to GeneScreen (New England Nuclear) according to the manufacturer's directions. A 1065-base-pair insert of pENOD2 (31) was prepared and nick-translated. Hybridization and washing conditions followed the GeneScreen manufacturer's protocol, washing at either 42°C or 65°C.

Microscopy. Excised nodule-like structures and roots were prepared for light microscopy using plastic sections (32).

Recovery of Bacteria from Nodules. Nodule-like structures were surface sterilized in 20% sodium hypochlorite for 2–3 min and washed three times, 5 min each, with sterile distilled water. The nodule-like structures were squashed whole in a minimal volume of sterile water and dilutions were plated on Luria broth agar without drugs. Recovered bacteria were replica-plated onto selective media for strain confirmation. Bacteria were also tested for nodulation ability following inoculation of axenically grown alfalfa seedlings.

RESULTS

Formation of the Pseudonodules. Structures resembling nodules were observed to develop on primary and secondary roots of alfalfa plants 2 weeks after applying either 5×10^{-5} M TIBA or 10^{-5} M NPA. The maximum number of pseudonodules appeared 3–4 weeks after the compounds were applied (Fig. 1). The pseudonodules were harvested at this time. NPA was more effective than TIBA; 60% versus 25% of the plants formed nodules. Bacteria recovered from selective media and tested on alfalfa seedlings did not induce nodules.

Nitrate did not inhibit pseudonodule formation in the presence of either TIBA or NPA. In many of the plants, the presence of the auxin transport inhibitors resulted in an increase in diameter of the elongation region of the primary roots. These roots superficially resembled the thick short root (*tsr*) response described for *Vicia sativa* roots (33).

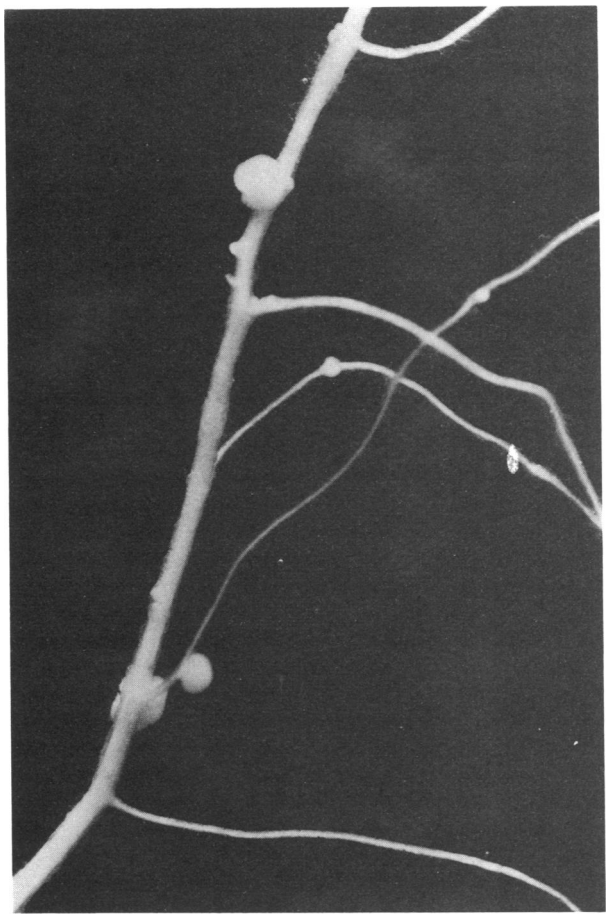

FIG. 1. TIBA application (5×10^{-5} M) induced the formation of white nodule-like structures on alfalfa roots. The nodules were harvested 3–4 weeks after treatment.

Histology. The anatomy of the NPA- and TIBA-induced pseudonodules was similar to that described for "empty" alfalfa nodules—i.e., nodules elicited by the *exo* mutants of *R. meliloti* (23). This was also true for nodules induced by *Agrobacterium* transconjugants containing *R. meliloti* nodulation sequences (3). A meristem was present (Fig. 2), but it was more diffuse than the meristem of the nodules induced by wild-type *R. meliloti*. Nodule cortex cells and cells of the central tissue were clearly distinguishable from each other, many of the central cells being filled with amyloplasts (Fig. 2). Distinct endodermal formation and vascular tissue differentiation were evident only in the proximal part of the pseudonodules. In this respect, the pseudonodules differed from the *exo* mutant and *Agrobacterium* transconjugant-induced nodules. In the latter, vascular tissue differentiates into the distal part of the nodule.

Gene Expression. We analyzed RNA isolated from the pseudonodules for the presence of ENOD2 and Nms-30 transcripts to see if the nodule-like structures were similar at the molecular level to the empty nodules induced by *exo* mutant *Rhizobium*. The expression of the Nms-30 nodulin gene, previously described as N-38 (25, 26), was determined by *in vitro* translation of nodule RNA followed by two-dimensional gel electrophoresis. The alfalfa ENOD2 gene expression was studied by RNA transfer blot analysis using the soybean pGmENOD2 clone as a probe (31).

The nodule-like structures elicited by either TIBA or NPA expressed the ENOD2 gene as did nodules induced by *exoH* or *exoB* mutants of *R. meliloti* (Fig. 3). The ENOD2 mes-

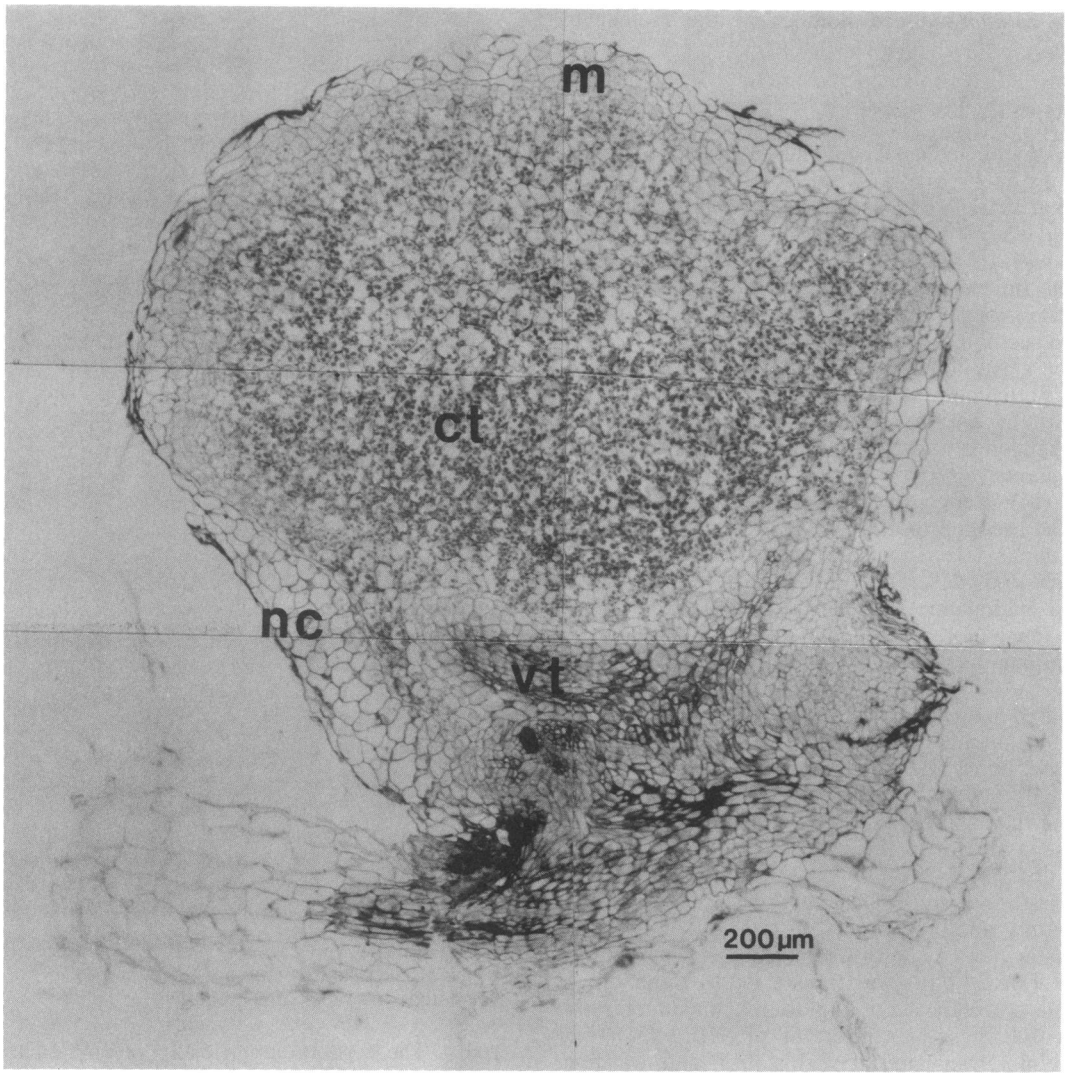

FIG. 2. Light microscope section (longitudinal) of a TIBA-induced nodule showing a histology similar to that described for other "empty" nodules, with a meristem (m) and a nodule cortex (nc) surrounding cells packed with amyloplasts of an uninfected central tissue (ct). The vascular tissue (vt) does not extend into the distal part of the nodule.

senger RNA was not found in RNA isolated from the *tsr*-like elongation zone of roots that were grown in medium containing either TIBA or NPA (Fig. 3).

Total RNA from pseudonodules of TIBA-treated plants was compared to RNA isolated from roots of untreated plants, wild-type nodules, and mutant *exoB*-induced nodules by *in vitro* translation and two-dimensional gel electrophoresis of the translation products. Fig. 4 *B–D* shows that the messenger RNA for Nms-30 was present in wild-type and *exoB* mutant-induced nodules and also in TIBA-elicited nodule-like structures (indicated by arrowheads). No translation product of comparable size and charge was found among the translation products of root RNA (Fig. 4*A*; position marked by a circle). Other nodulins, such as Lb (indicated by arrows in Fig. 4*B*), were present only in the wild-type-induced nodules.

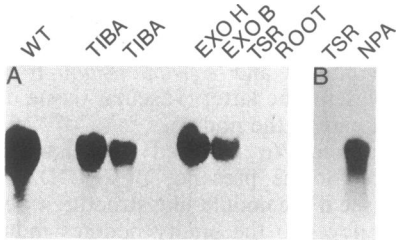

FIG. 3. Expression of the ENOD2 gene. Following treatments described in the text, RNA was isolated from alfalfa nodules and roots and probed with pGmENOD2. (*A*) RNA from nodules induced by wild-type (lane WT) and mutant (lanes exoH and exoB) *R. meliloti*, from nodule-like structures induced by TIBA (two separate experiments), from root tips that expanded radially (lane TSR) in the presence of TIBA, and from untreated alfalfa roots. (*B*) RNA from swollen root tips (lane TSR) grown in the presence of NPA and from nodule-like structures induced by NPA.

DISCUSSION

We have found that nodule-like structures initiated by auxin transport inhibitors on alfalfa roots are structurally similar to root nodules induced by *Rhizobium* exopolysaccharide mutants. These results confirm and extend earlier reports on the same subject (refs. 21 and 22; J.G.T., unpublished results). We have also determined that in these nodule-like structures two genes, ENOD2 and Nms-30, are expressed. Transcripts for these genes have previously been found to be present only in *Rhizobium*-induced root nodules.

Previously, we used histological criteria to describe the empty nodules induced by *Rhizobium exo* mutants and *Agrobacterium* transconjugants carrying the *nod* genes of rhizobia (3, 23). The nodule-like outgrowths initiated by the

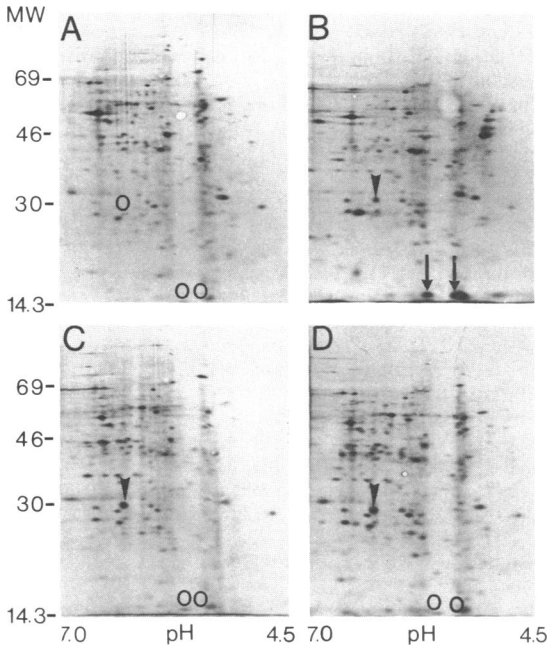

FIG. 4. *In vitro* translations of total RNA isolated from untreated roots (*A*) and wild-type-induced nodules (*B*). The arrowhead points to Nms-30 and arrows point to Lb. (*C*) Nodules induced by *exoB* mutant *R. meliloti*. (*D*) Nodules elicited by TIBA. Nms-30 (arrowhead) is present in both *C* and *D*, but Lb is absent (open circles).

two growth regulators, TIBA and NPA, resembled these empty nodules in histological organization, with one major exception. We did not observe differentiated vascular tissue in the distal portion of the nodule-like structures.

A characteristic feature of nodules induced by rhizobia is expression of genes known as the nodulin genes. Some of these are involved in nodule formation and others in nitrogen fixation. We believe that the expression of the two nodulin genes, ENOD2 and Nms-30, can serve as valid criteria to distinguish the chemical-induced outgrowths as nodules for the following reasons. The early nodulin ENOD2 is expressed in all legume nodules studied to date (34). In addition, *in situ* hybridization studies show that ENOD2 is expressed exclusively in the inner cortex of the nodule in both determinate (soybean) and indeterminate (pea, alfalfa) nodules (C. van de Wiel and T.V.B., unpublished results; A.M.H., unpublished results). In these studies, messenger RNAs for ENOD2 were not found in the nodule meristem. This suggests that the expression of the ENOD2 gene reflects the differentiation of one of the tissues formed in true nodules. The early nodulin, Nms-30, is expressed abundantly in the wild-type and *exo* mutant-derived nodules of alfalfa. The role of Nms-30 in nodule development, however, is not clear. The expression of the ENOD2 and Nms-30 genes, as well as the histology of the root outgrowths, indicates that these nodule-like structures are indeed nodules.

The expression of the two nodulin genes in the absence of *Rhizobium* suggests that the two early nodulin genes may be developmentally, rather than symbiotically, regulated. However, expression in tissues other than nodules has not been demonstrated. The expression of the ENOD2 and Nms-30 genes is characteristic of empty alfalfa nodules (24–26). This suggests that *Rhizobium* is able to control the plant's developmental program, resulting in the expression of nodulin genes. The expression of nodulin genes and the histological resemblance to *Rhizobium*-induced nodules leads us to speculate that *Rhizobium* may initiate nodule development by changing the cytokinin/auxin ratio of root cortical cells. This then causes cellular division. *Rhizobium* is known to secrete cytokinins. One possibility is that the *nod* genes encode products that directly or indirectly influence cytokinin secretion. This secretion would perturb the plant's endogenous cytokinin/auxin ratio. Experiments by J. B. Cooper and S. R. Long (personal communication) support this interpretation. They observed that spot-inoculating alfalfa roots with a *nodA* mutant of *R. meliloti*, carrying the *tzs* (*trans*-zeatin synthesis or secretion; ref. 35) gene of *A. tumefaciens*, results in the formation of empty nodules not infected by *Rhizobium*. However, it is not known whether the nodulin genes, ENOD2 and Nms-30, are expressed in the nodules induced by these transconjugants.

Another possibility is that *Rhizobium* controls the auxin/cytokinin ratio by producing inhibitors of auxin synthesis, transport, or action. Jacobs and Rubery (36) reported that various plant flavonoids—e.g., apigenin—compete with synthetic auxin transport inhibitors. It is well documented that flavones regulate *nod* gene activity (9–11). It is feasible that *Rhizobium* synthesizes product(s), perhaps modified flavones, that function as auxin transport inhibitors.

The initial stages of nodule formation, root hair deformation and the induction of cortical cell divisions, are mediated by wild-type *nod* genes. We have shown that NPA and TIBA, two auxin transport inhibitors, induce cortical cell divisions and the development of nodules, which in turn express two nodulin genes. Thus, TIBA and NPA are able to substitute for the activity of compounds made following the induction of *Rhizobium nod* genes.

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